



A newly emerged focus of zoonotic cutaneous leishmaniasis in South-western Iran

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ABSTRACT

Leishmaniasis is rising in many countries, including Iran, due to climate change, refugee crises, urbanization and etc. The aim of this study was to explore the epidemiology, extent and identity of *Leishmania* species in a newly emerged focus in Abadan County, Ilam Province, South-western Iran. This study was performed as a descriptive cross-sectional study by a systematic house-to-house approach. The *Leishmania* species was identified by RFLP-PCR and sequencing. Altogether, 46799 individuals consisting of 22907 (48.9) female and 23892 (51.1%) male were interviewed and physically examined for the presence of skin lesions. Overall, the incidence rate was 0.34% (n = 160). All age groups were affected and the incidence rate was the highest in < 10 years of age group (0.49%) and the lowest in > 50 years old individuals (0.15%), although there was no significant difference regarding the sex and age. The majority of patients had one lesion (47.5%) on hands (56%) and most of the cases occurred in Abadan city (54%) in summer. Based on the RFLP-PCR analysis, all the *Leishmania* isolates were *L. major* of single genotype. A newly emerged focus of zoonotic CL caused by *L. major* occurred in South-western of Iran. Multiple risk factors created this epidemic area. Further studies on the vector and reservoir are crucial needed to provide evidences to select the prophylactic and therapeutic measures for future control strategies.

1. Introduction

Leishmaniasis transmission occurs in 5 continents. An estimated prevalence of 12 million people in nearly 100 countries and territories with leishmaniasis and over 350 million are at high risk. Moreover, approximately an incidence of 1.5–2 million new cases occurs per annum worldwide. Cutaneous leishmaniasis (CL) is largely neglected and hidden in the endemic countries as well as numerous cases remain unreported. Among different types of leishmaniasis CL is the dominant presentation with 70–75% prevalence at the global range, more importantly in the Eastern Mediterranean Region, Latin America, Africa and Indian Subcontinent. Over 90% of the CL cases occur in seven countries including Afghanistan, Iran, Algeria, Brazil, Saudi Arabia, Peru and Syria [1–3].

Currently, the number of patients who suffer from CL in developing countries, frequently in tropical and subtropical areas have been rising

[1,2]. Recent global documents show that CL has been rising and expanding to new foci as documented in Syria, Morocco, Brazil, Iraq, Afghanistan, Pakistan, Libya, Israel, and Iran [3–7]. Various risk-related factors play a central role in provision of favorable condition for propagation of sand flies, transmission of new cases, creation of endemic foci and expansion of new geographical ranges such conditions include urbanization and environmental changes, population movements, drug resistance, international conflicts, natural disasters, immune suppression due to HIV/AIDS and increased travels and trades [8–11]. Such determinants can lead to sudden or gradual induction of outbreaks in new geographical locations [3]. A major increase in the spread of the disease is attributed to the refugee crises in the Middle East and North Africa, over the last decade, particularly due to movement of millions of Syrian, Iraqi and Afghani refugees [5,12].

In Iran, zoonotic CL (ZCL) and anthroponotic CL (ACL) caused by *Leishmania major* and *L. tropica*, respectively are endemic in 18 out of 31

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Provinces with an average of 20,000 official cases each year. Although, the actual figure is significantly greater than that reported [13]. Over the past decades, more than 15 recorded epidemics of CL and numerous unreported ones occurred in Iran. Anthroponotic CL (ACL) due to *L. tropica* epidemics occurred most frequently in Kerman Province [14–16], while other epidemics due to ZCL have been reported in the different Provinces of the country [13,17–22]. The present study was aimed to explore a newly emerged ZCL epidemic in Abadan County in Ilam, South-western Iran. The epidemiology, extent of its spread, probable risk factors and the identity of the causative agent were determined by direct smear microscopy, conventional PCR and RFLP-PCR. Also sequencing was performed for phylogenetic analysis.

2. Materials and methods

2.1. Ethical issues

This study was approved by Ethics Committee of Kerman University of Medical Sciences under Ethics Committee no. IR.KMU.REC.1395.269. Before the survey began, several preliminary meetings were held with authorities, health personnel and community members. Issues including various tasks, cooperation, referral and diagnosis of the cases, were discussed. Written informed consents of the CL cases were obtained before smear preparation. Patients were referred to the clinics and district hospital for treatment free of charge. The patients' demographic and clinical data were kept confidential.

2.2. Study area and population

This study was carried out in Abadan County, Ilam, South-western of Iran for a period of one year, starting from March 2015 up to February 2016. The county is located between 32° and 59°E and 47° and 25°N which covers 2385 km² areas. The County consists of 48 villages; 10 villages were affected and the remaining 38 villages remained unaffected. The region is located 165 km in South-east of the city of Ilam, the center of Ilam and is 880 m above the sea level. The climate is moderate to warm in the summer (31°C - 42°C) and cool in the winter (3°C - 9°C). The population is about 46,799 (Fig. 1). A massive earthquake struck the County with a magnitude of 6.2 on the Richter scale in 2014. Currently, several new agricultural projects for growing different

vegetable and crops have been developed in the area.

2.3. Sampling and interview

A cross-sectional descriptive and systematic house-to-house census survey was performed by a team of health personnel. Each suspected individual was undertaken a thorough physical examination. Those subjects who displayed skin lesions were considered as probable cases and referred to a designed CL diagnostic laboratory for detection and identification of the causative organism.

2.4. Smear preparation

A scalped with blade was used to take the smear from the indurated margin of the cutaneous lesions. For each case two direct slide smears were prepared. The smears were methanol-fixed, Giemsa-stained and air dried. The slides containing smear were microscopically examined for the presence of amastigote stage (Leishman bodies). Before direct smear preparations a questionnaire was completed for each patient. Demographic data (gender, age and residence) and clinical status (number, location and the onset of lesions) were recorded. The other slide was used for molecular identification.

2.5. Molecular identification

DNA was extracted from amastigotes of the direct smear preparations using DNeasy Blood & Tissue Kits (Qiagen, Germany) based on the manufacturer's protocol. The extracted DNA was stored at -20°C for future PCR amplification.

The samples were analyzed for ITS1 PCR using primers: LITSR: 5'-CTTGCAATTTCCGATG-3' and L5.8S 5'-TGATACCACTTATCGC-3' which was previously described by Schönian et al., 2003 [23] with some modification based on Mohammadi et al., 2017 study [24]. PCR reaction was carried out with totally 25 µl of mixed reagents based on the described PCR conditions. Finally, 5 µl of the PCR products was electrophoresed and visualized on 1.5% agarose gel electrophoresis followed by ethidium bromide staining for confirmation of PCR-specific amplification. Restriction fragments analysis was done with 10 µl of PCR product which was digested with *HaeIII* enzyme, according to the manufacturer's instructions. The restriction fragments were analyzed on

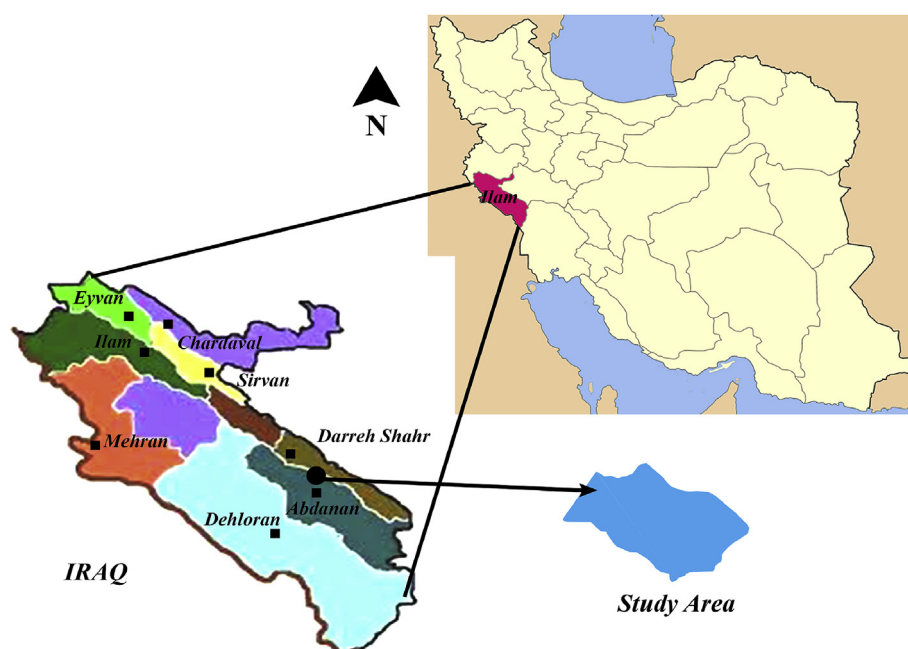


Fig. 1. Map of Iran displaying the study area where newly emerged focus of zoonotic cutaneous leishmaniasis occurred.

3% agarose gels by electrophoresis and visualized by staining with ethidium bromide under UV light.

2.6. Sequencing

Randomly 15 positive samples were selected and sequenced by MacroGen Company (South Korea). Sequences were trimmed with sequence scanner ver.1 (Applied Biosystems, USA). Analyses of multiple sequence alignments were done using the programs BioEdit ver.6 and phylogenetic tree was constructed by MEGA ver.7 [25,26].

2.7. Statistical analysis

Data was entered into a computer. SPSS statistical software (Ver16, Chicago, IL, USA) was used. Chi-square test was performed to determine significant differences between demographic and clinical data in addition to the infection rate. Statistical significance was set at $p < 0.05$.

3. Results

Overall, 46799 residents in Abadan County, consisting of 23892 (51%) males and 22907 (49%) females aged 1–83 years (mean; 25 ± 17 years) were interviewed and clinically examined for the presence of skin lesions (Table 1). The incidence rate (new cases) of active lesions was 0.34%, including 160 cases; 93 males (0.41%) and 67 females (0.33%), although there was no significant difference among the gender. Skin lesions were active sores and patients showed no scars which demonstrate an ongoing emergence of a new CL epidemic area. All age groups were affected; CL patients < 10 years old displayed the highest (0.49%) while > 50 years old age group showed the least rate of infection (0.15%). Although, there was no significant difference between the children (< 10 years old) and other age groups. Most of the skin lesions were on hands (56%), followed by legs (19.4%), face (10.7%), trunk (1.2%) and the remaining (12.7%) were on the other parts of the body (Fig. 2). Multiple lesions between hand-legs, hands-face and hands-trunk (2%, each), legs-trunk (1.2%) and legs-face (0.7%), were observed. The majority (47.5%) of patients had single lesion, 25.5% two lesions and 27% ≥ 3 lesions (Fig. 3) (See Table 2).

Monthly incidence of the cases is presented in (Fig. 4). Most of the CL lesions occurred in June (25.6%), followed in May (16.9%), July (15%) and to lesser extent in other months; even though, the cases occurred throughout the year. The majority of patients were infected in the city of Abadan, the most populated place among the affected areas. The incidence of CL cases in Haftcheshmeh and Shahrak were similar (10.6%, each), followed by Srabbagh (10%), Chamkabood (7.5%), poshtghaleh (2%), then in Sarpeleh, Mahooteh and Gandab (1.2%, each) and the least in Zhivar (0.7%) (Fig. 5).

Based on the PCR-RFLP analysis all isolates amplified a 350bp fragment for specific ITS primers and displayed 134 and 200 bp bands after enzymatic restriction experiment, which respectively corresponding to *L. major* species. (Fig. 6A, Fig. 6B).

Nucleotide sequence has been submitted to the GenBank database

Table 1

Sex and age distribution of affected population in Abadan County, South-western Iran.

Age (year)	Female	Male	Total
	No (%)	No (%)	No (%)
< 10	3472 (15.1)	3748 (16)	7220 (15.5)
10–30	8626 (38)	9118 (38)	17744 (38)
31–50	7175 (31)	7085 (30)	14260 (30.5)
> 50	3634 (15.9)	3941 (16)	7575 (16)
Total	22907 (100)	23892 (100)	46799 (100)

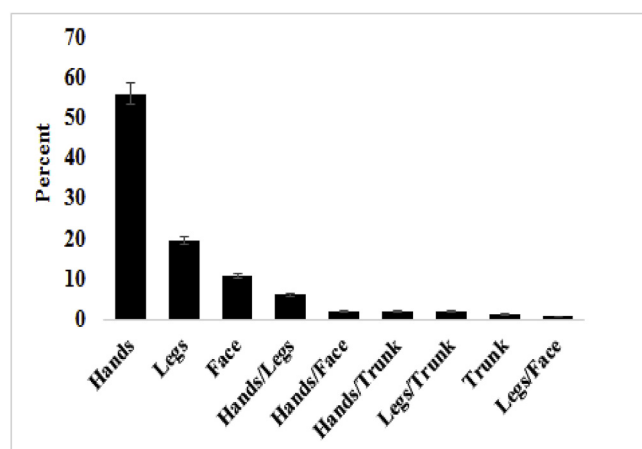


Fig. 2. Anatomical location of lesions in patients with zoonotic cutaneous leishmaniasis in Abadan County, South-western Iran.

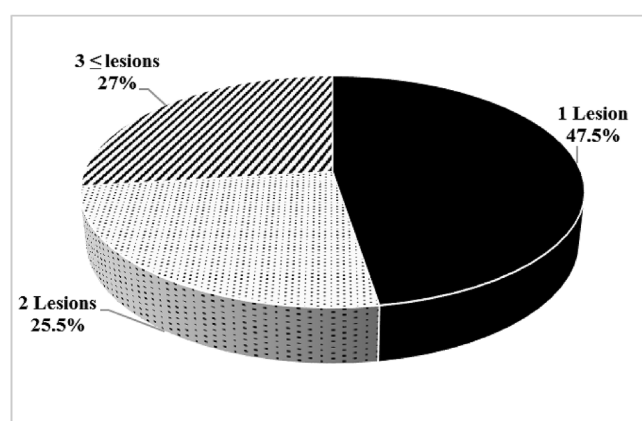


Fig. 3. Number of lesions in patients with zoonotic cutaneous leishmaniasis in Abadan County, South-western Iran.

Table 2

The incidence of zoonotic cutaneous leishmaniasis patients by gender and age groups in Abadan County, South-western Iran.

Age (years)	Female	Male	Total
	No (%)	No (%)	No (%)
< 10	17 (0.48)	17 (0.45)	34 (0.47)
10–30	22 (25)	41 (0.44)	61 (0.34)
31–50	27 (37)	28 (0.39)	55 (0.38)
> 50	3 (0.08)	7 (0.17)	10 (0.13)
Total	67 (0.29)	93 (0.38)	160 (0.34)

with accession number MF522259 (Fig. 7).

All PCR products sequences which were the same and phylogenetic analysis was confirmed PCR-RFLP results as *L. major*. However, Blast the consensus sequences show the maximum identity with other *L. major* isolated in Iran.

4. Discussion

Cutaneous leishmaniasis is significantly growing and imposing major social and health morbidity in Iran and over 98 countries of the world [2]. The present study clearly indicates that how confounding risk factors could collectively lead to sudden emergence of a new ZCL outbreak in a place with no previous history. All the skin lesions were active providing further evidence that this emergence is a new occurrence.

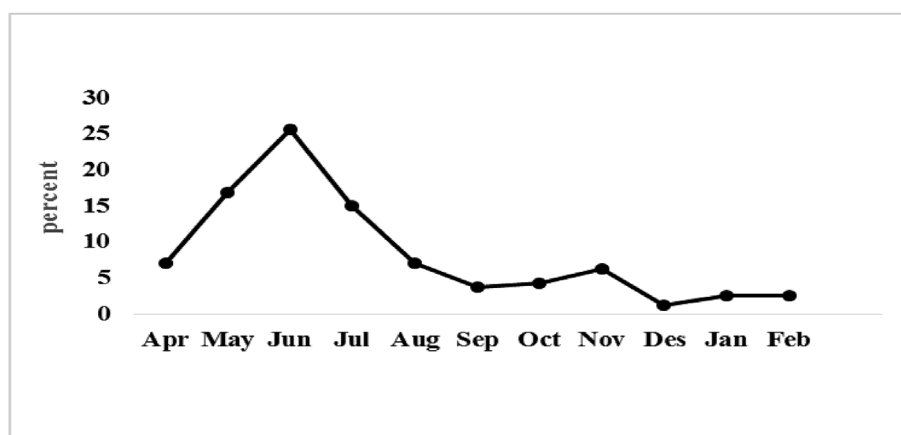


Fig. 4. Monthly incidence of zoonotic cutaneous leishmaniasis patients in Abadan County, South-western Iran.

Both gender and age groups were equally affected and there was no significant difference among various groups. Although, younger individuals (< 10 years old) displayed higher infection rate than the older subjects (> 50 years of age). It is well known that in epidemics due to the non-immune status of the subjects, females and males in all age groups are susceptible in contracting the infection. However children because of higher exposure, less disciplined and for incomplete dressing are attracted and bitten more frequently by the vector. Hence, the female sand flies take advantage of such conditions for blood meal during the nocturnal period [9]. The predilection of age and sex for affliction of CL in emerging outbreaks is not well known. Different characteristic manifestations related to demographic features have been reported. These variabilities can be behavioral, cultural and differences in *Leishmania* species.

In this study, hands, legs and face collectively represented 86.1% infection which were the common site of involvement. The above demographic presentations are consistent with *L. major* causing ZCL in other emerging foci of Iran [21]. The number of lesions are also indicative of ZCL epidemic in this region. All the cutaneous lesions were active sores proving further evidence that the site is a new emerging focus.

In an inquiry to explore the main reasons for such a new occurrence, no single and clear evidence was found. However, several combinatory risk factors could have played roles in the sudden abrupt of this outbreak: (i) Neighboring Counties such as Dehloran and Mehran had already been affected [27,28], notably after Iraq-Iran war, where ZCL was previously established. (ii) Development of new agricultural projects for growing crops in the County (iii) The

2014 devastating earthquake of Abadan also created a suitable condition for propagation and breeding of the vector and ultimately transmission of the human cases. (iv) Recent expansion of villages and resultant ecological changes favored the attraction of the main reservoir host, small gerbils; although, so far no investigation has been performed. The above determinants possibly played a synergistic action in creation of this new emerging focus in South-western Iran.

Such conditions gradually or suddenly caused many ACL outbreaks due to *L. tropica* in several rural and urban areas in the province of Kerman, following the 2003 earthquake in Bam [29]. However, ZCL caused by *L. major* as the predominant epidemiological form represented higher number of registered CL epidemics in Iran [13,17,19,21,30]. Similar risk factors leading to CL epidemics were reported from other countries [9,31,32].

Traditional methods based on intrinsic criteria for detection of responsible *Leishmania* species mainly in mixed foci are not sufficient [23]. In the present study a combination of reliable and sensitive molecular methods utilizing ITS1 gene and sequencing approaches were employed. Unlike traditional epidemiological and clinical characteristics, the present molecular methods as gold standards are universally used in precise discrimination of the causative agent [23].

The majority of the lesions occurred during the late spring and mid-summer in Abadan. It seems that the peak of activity of the female sand flies started sometime in April where the exposure and the transmission of infection occurred. This reflects 2–4 months of incubation to complete development of lesions in the host [33]. Most of the cases occurred in Abadan city and suburb and three surrounding

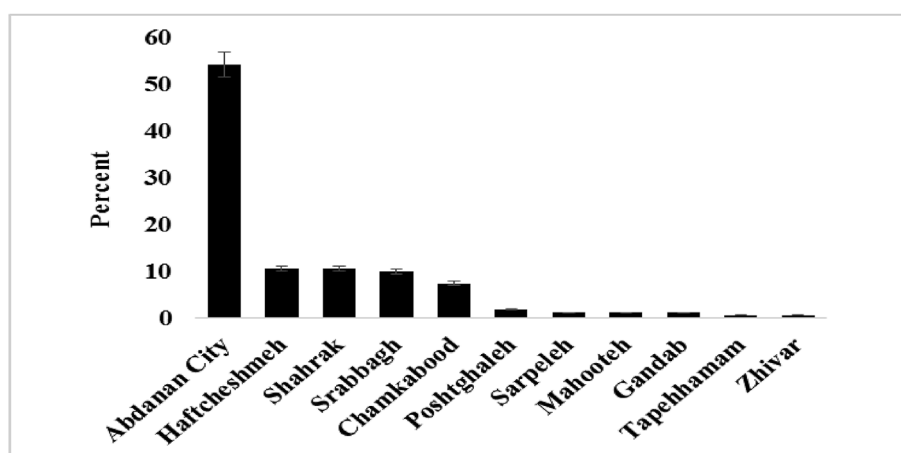


Fig. 5. Distribution of zoonotic cutaneous leishmaniasis patients in Abadan city and surrounding villages in Abadan County, South-western Iran.

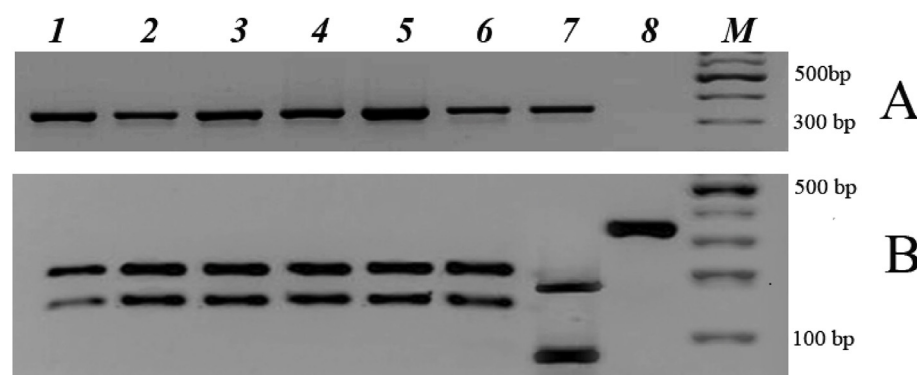


Fig. 6. Electrophoresis of ITS1-PCR. A) The conventional PCR-based detection of *Leishmania major* amastigotes in representative smear preparations from Abdanan County. The amplified 300–350 bp product of specific primers. Positive control sample *L. tropica* (lane 1) and *L. major* (lane 2), positive test samples (lanes 3–7) and non-template control (lane 8). A 100 bp DNA size marker (Lane 9). B) Three-percentage agarose gel electrophoresis of ITS1-PCR restriction fragments length polymorphism. Positive test samples (lanes 1–6), *L. tropica* control sample (lane 7) and *L. major* PCR product (lane 8). A 100 bp DNA size marker (Lane 9).

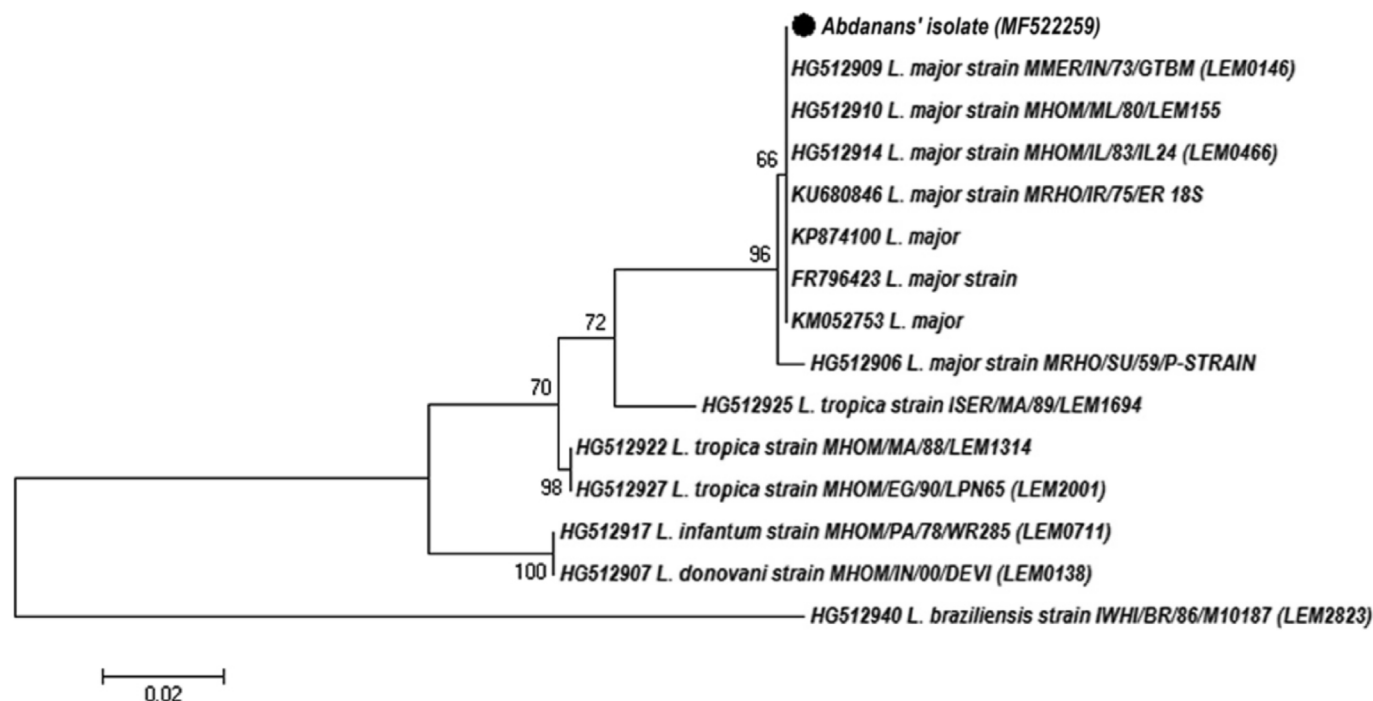


Fig. 7. Molecular phylogenetic analysis of partial ribosomal RNA. The evolutionary history was predicted by using the Maximum Likelihood method based on the Kimura 2-parameter model. The tree was constructed by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The best nucleotide substitution model was tested and a discrete gamma distribution was used to model evolutionary rate differences among sites. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7. A representative sequenced Askari isolate displayed high similarity to other *Leishmania major* strains particularly last Iranian's isolate KU680846.

villages (Haftcheshmeh, Shahrak and Sarab-Bagh) near the previous endemic foci in Mehran and Dehloran cities in close border with Iraq [27,28,34]. The sequenced isolates demonstrated only one homogenous strain of the same genotype. This finding is consistent with the isolates reported from other parts of Iran [35].

A sudden abrupt of a ZCL outbreak occurred in Abdanan County in South-western of Iran, in close border to Iraq and Dehloran and Mehran in Ilam province where the disease was previously endemic. Multiple risk-related factors including cross-border movements, massive earthquake prior to the onset of the disease occurrence and recent development of agricultural projects possibly created a suitable condition for breeding of the vector and occurrence of this epidemic disease. All age and sex groups were affected. Based on the clinical presentations and molecular markers *L. major* was the only causative agent. Further studies on the vector and reservoir are crucial to provide evidences to select the prophylactic and therapeutic measures for planning future control strategies.

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